Pathway Analysis and Optimization in Metabolic Engineering

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CAMBRIDGE
UNIVERSITY PRESS

PUBLISHED BY THE PRESS SYNDICATE OF THE UNIVERSITY OF CAMBRIDGE
The Pitt Building, Trumpington Street, Cambridge, United Kingdom

CAMBRIDGE UNIVERSITY PRESS
The Edinburgh Building, Cambridge CB2 2RU, UK
40 West 20th Street, New York, NY 10011-4211, USA
477 Williamstown Road, Port Melbourne, VIC 3207, Australia
Ruiz de Alarcón 13, 28014 Madrid, Spain
Dock House, The Waterfront, Cape Town 8001, South Africa

http:// www.cambridge.org

First published 2002

Printed in the United States of America

Typefaces Sabon 10/13 pt. and Franklin Gothic *System* LaTeX 2_ε [TB]

A catalog record for this book is available from the British Library.

Library of Congress Cataloging in Publication Data

Torres, Néstor V., 1958–
Pathway analysis and optimization in metabolic engineering / Néstor V. Torres, Eberhard O. Voit.
p. cm.
Includes bibliographical references and index.
ISBN 0-521-80038-2
1. Biochemical engineering – Mathematical models. 2. Mathematical optimization. 3. System theory. I. Voit, Eberhard O. II. Title.
TP248.3 .T67 2002
660.6′3 – dc21 2002071511

ISBN 0 521 80038 2 hardback

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CHAPTER ONE

Target: A Useful Model

CRITERIA FOR MODEL SELECTION

Before we get lost in the technical details of manipulating functions, approximating complicated phenomena, or designing and analyzing models, it is useful to establish more clearly what exactly our target is. At a superficial level, this is easily stated. Our target is a mathematical model of the biotechnological phenomenon of interest, and this model should be valid, yet convenient for analysis, manipulation, and optimization. Once we have such a model, we can screen hypotheses and perform test runs on the computer, which is much faster and cheaper than implementing and executing the actual experiments in the lab.

While the target is obvious, the difficulty is that no unique, optimal model entirely satisfies all items on our wish list. Why is that? The complications begin with the question of validity. What is a valid representation of a particular phenomenon? Although initially surprising, the question of validity is not something absolute. Instead, validity depends heavily on the purpose of the model analysis. A model for studying the aerodynamics of a butterfly will normally not account for the color patterns of its wings, and that is probably a valid omission. By contrast, the coloration may be crucial for ecological questions of camouflaging and predation by birds.

As a familiar, yet illustrative example, consider the growth of a bacterial population (Thornton 1922), as discussed by Lotka (1924, pp. 70–1; see Table 1.1 and Figure 1.1).

The symbols in the figure show the observed size of the bacterial colony over time, and the line is the graph of the logistic function

$$S(t) = \frac{0.2524}{\exp(0.005125 - 2.128t)}. \tag{1.1}$$

Inspection of Figure 1.1 suggests that the function $S(t)$ fits very well. Nonetheless, as the table indicates, there are discrepancies between observed and computed values. Can the logistic function be considered a valid representation, even though it underestimates the true colony size at day 1 by almost 30%? There is no definite answer.

Table 1.1. Observed and Calculated Growth of a Bacterial Population (Adapted from Lotka 1924)

Age of Colony (Days)	Area in cm^2	
	Observed	Calculated
0	0.24	0.2511
1	2.78	2.0324
2	13.53	13.0761
3	36.3	37.0479
4	47.5	47.3930
5	49.4	49.0231

Obviously, the function captures the saturating trend in colony growth and returns sizes reasonably close to those observed. Furthermore, some error no doubt exists in the data, which might account for the inaccuracies. After all, even in the ideal case of perfectly circular colonies, the calculated and observed colonies at day 1 differ by merely one-twentieth of an inch in radius. This simple analysis suggests that good data fit alone is not a reliable criterion for the validity of a model.

Another aspect of the same example is whether the logistic function "explains" anything. On one hand, the function allows us to make relatively accurate predictions about the size of the colony between observations and beyond the observation period. For instance, the function would have predicted the colony size at day 5 quite accurately, even if it had not been measured. This power of prediction implies that the model provides a certain degree of explanation. On the other hand, the parameter values of the function (0.2524, 0.005125, and −2.128) are not meaningful, or even measurable quantities that could be obtained from the bacterial colony itself. If we had a new colony, these parameter values would most probably not be optimal. Also, the simple logistic "model" does not capture any of the biological phenomena that underlie the growth of the bacteria, their biochemistry, or their physiology. If we wanted to predict the growth of the same type of colony under the influence of a growth inhibitor, this model alone would not be too helpful.

In conclusion of the present discussion of validity, we must distinguish what is important to capture in a given model and what can be ignored. In the biotechnological

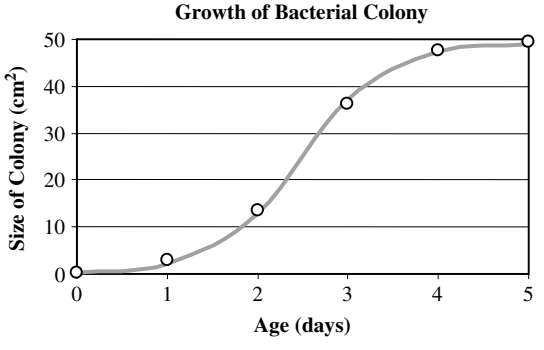

Figure 1.1. Growth of a colony of bacteria over a time period of five days. Circles indicate observations, the line is the graph of a logistic function (see text for details).

setting of a microbial culture or chemostat, typical variables of interest are metabolites, enzyme activities, fluxes, and controls such as pH, temperature, oxygen content, cofactors, and of course substrates. The physical shape of the involved molecules is clearly of importance for the enzyme-catalyzed reactions to proceed, but for typical batch processes it is often of secondary interest. Thus, returning to the issue of identifying our target, we envision a model that allows us to ask questions about changes in fluxes, metabolite concentrations, microbial population sizes, the relative importance of fluxes that funnel material through the system of metabolic pathways, and the effects of substrates and modulators. The model should enable us to map relevant observations into a mathematical realm, which would then allow us to execute "virtual experiments" and explore hypothetical scenarios.

Validity is certainly important, but it is not the only criterion in the selection of a model. A second requirement for a good model is its mathematical tractability. Only a model that permits effective evaluation, preferably both algebraically and computationally, has the potential of becoming a general tool in an applied science. We shall see in the next sections that some of the traditional models of enzyme kinetics are very useful for studying individual processes, but that they can become mathematically unwieldy in a network of just a few pathways.

If we had no history of modeling biochemical phenomena, our first stab at a useful model would probably be some linear system. The reason for this choice would be that no other branch of mathematics offers as rich a repertoire of theorems, methods, and tools as linear mathematics, and validly representing our phenomena of interest with linear methods would be half the battle. However, it has been said that focusing on linear functions within the huge realm of nonlinearities is like dividing the animal kingdom into elephants and non-elephants. Indeed, if one goes by the number of all possible mathematical structures, linear models are negligible. Even so, it is still often well worth considering linear systems, because they have very many unique and desirable features. This will become apparent throughout the book.

The drawback with linear functions in a biotechnological context is that they are often simply not appropriate descriptions of natural phenomena. For instance, essentially all processes in living organisms saturate if the dependent variable becomes very large. The growth of a population may be linear or exponential (i.e., linear in the logarithm) for small population sizes, but eventually the growth cannot continue unabated and the growth function ultimately flattens. Another limitation of linear models is their inability to represent stable oscillations that return to their original dynamics after a small perturbation. There are many oscillatory phenomena in biology, and many of them are stable in this sense. The inability of linear models to capture these oscillations is therefore a drawback. The same applies for chaotic responses, which also require nonlinear descriptions.

If linear functions are not justified, one has three options for model selection. First, one could use linear functions anyway and accept the consequent inaccuracies. This may sound like too drastic a simplification, but much of engineering is based on linear systems, and the enormous accomplishments of engineering attest to the validity of this approach. However, a crucial difference between engineering and

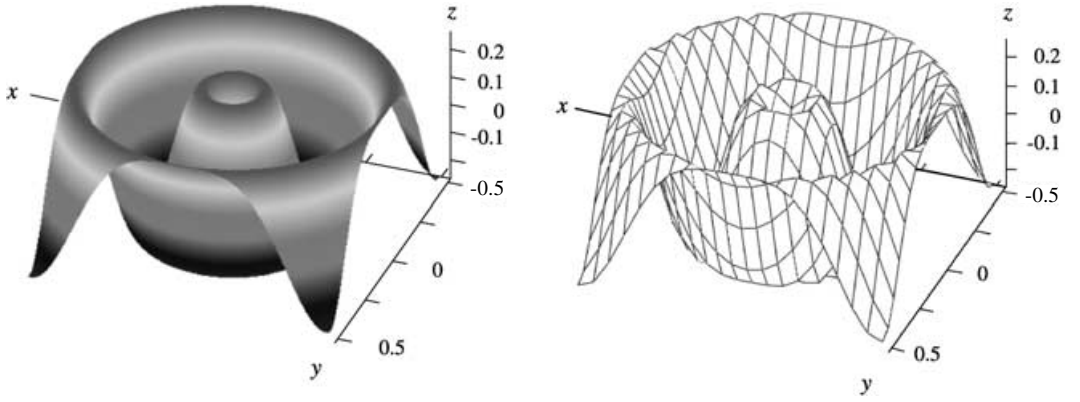

Figure 1.2. Function $z = 0.25 \cdot \sin[5\pi(x^2 + y^2)^{1/2}]$ of two independent variables, x and y, and its "wiremesh" representation, which corresponds to a two-dimensional piecewise linear approximation, if each quadrilateral is dissected into two triangles.

biology is that engineers are often in a position to design systems according to their own specifications, and these may include linear response functions. Biologists, by contrast, must live with what nature presents, and that is usually nonlinear.

The second option is using a piecewise linear representation. To represent a function in this fashion, one replaces it with sufficiently many small linear pieces, and the analysis shifts from piece to piece, depending on the value of the dependent variable. The analogous procedure may be applied to higher-dimensional functions (Figure 1.2).

Obviously, the smaller the individual pieces, the closer the agreement between the piecewise linear model and the modeled nonlinear reality. But, of course, there is again a drawback: smaller individual pieces require more breakpoints or "breaklines" between adjacent pieces and thus complicate the computational implementation and analysis.

The third option is to accept and confront the nonlinearities as they appear. The challenge here is that the true structure of the nonlinearity is seldom known. Even smooth and nearly error-free data that show a nonlinear trend do not uniquely identify the mathematical form of the underlying function. As an example, consider fabricated "data" with modest experimental error from a simple saturated process (Figure 1.3). Without further information, these data could be adequately modeled by a variety of functions, such as a shifted Hill function $f_1(x)$, an arctangent $f_2(x)$ or logistic function $f_3(x)$, or even a statistical distribution function $f_4(x)$. The function $f_4(x)$ is the cumulative of the normal distribution $N(1.7, 1.2)$, multiplied with 1.85. Similarly, other cumulative frequencies, like Student's t-distribution, could be used to model the data. Even the sine function $f_5(x) = 0.8 \cdot \sin(0.82 \cdot x + 4.8) + 1$ fits rather well.

The point of this comparison is that the criterion of a close data fit is rather weak and unreliable. In particular, a good data fit alone does not provide strong guidance for model selection. Sorribas, March, and Voit (2000) reached a similar conclusion in the context of identifying the best-fitting statistical distribution for a given data

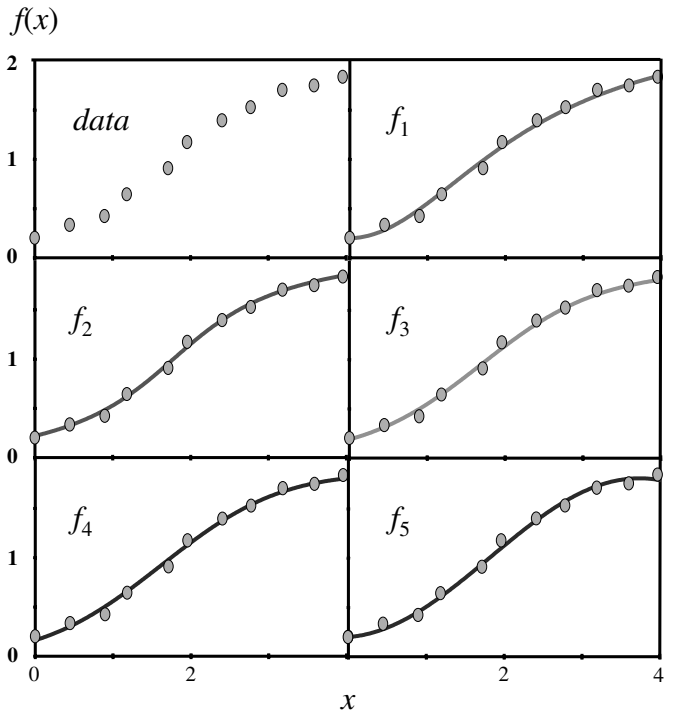

Figure 1.3. Numerous functions provide pleasing fits to the fabricated data in the top left panel. Shown here are a shifted Hill function ($f_1(x) = \frac{2.2x^2}{2.3^2+x^2} + 0.2$), an arctangent function ($f_2(x) = 0.77 \cdot \text{arctg}(0.9x - 1.6) + 1$), a logistic function ($f_3(x) = \frac{1.9}{1+\exp(2.2-1.3x)}$), a stretched normal cumulative ($f_4(x) = \frac{1.85}{\sqrt{2.4\pi}} \int_{-\infty}^{x} \exp(-\frac{1}{2.88}(u - 1.7)^2)\, du$), and a sine function ($f_5(x) = 0.8 \cdot \sin(0.82 \cdot x + 4.8) + 1$). The data fit alone is not sufficient to prefer any of these functions to an alternative.

set. They drew random numbers from a given distribution and showed that in a high percentage of cases these data were better fitted by a different distribution than the original that had been used to generate the random numbers.

With no reliable guidance from a graph of data, one has two choices of model selection. One may use a black-box model that fits the data as the logistic function fits the measured bacterial growth data and the functions f_1 through f_5 fit the manufactured data in Figure 1.3. A function found this way is often simple and robust, but its explanatory value is limited, as discussed previously. The alternative approach is to search for valid descriptions of the underlying processes. In most biological phenomena, these processes are not isolated but highly interconnected, and if we are to capture the essential features of these phenomena, we must find effective mathematical ways of representing systems.

The following sections describe some approaches to developing explanatory candidate models. We begin with a very brief review of the derivation of the best-known biochemical model, the Michaelis–Menten rate law. This rate law has proven extremely useful for the analysis of individual reactions in vitro, and thousands of articles deal with the characteristic parameters of this rate law, the Michaelis constant

K_M, and the maximal velocity of the reaction, V_{max}. Although widely used, the Michaelis–Menten rate law has serious disadvantages. Again, they fall in two categories: validity and tractability. Some generalizations of the original rate law overcome problems with the validity of the underlying assumptions. However, these generalizations exacerbate the challenges of tractability. Ultimately, rate laws of this type lead to so many mathematical and biological challenges that we have to search for other solutions.

The proposed solution in this chapter, and indeed throughout this book, is to use power-law approximations of processes. The advantages of these approximations include a relatively wide range of validity, mathematical justification, a good fit to observations, and the feature of scale-invariance, which has also been called the *telescopic property* (Savageau 1979a, 1985). Whether the system is small or large, whether a process involves two or 200 variables, whether the model addresses a phenomenon at a low or a high level of biological organization, the mathematical structure of these representations remains the same. This scale-invariance has tremendous implications, from both a conceptual and a practical point of view.

MODELS OF BIOCHEMICAL PROCESSES

Fortunately, our search for the best mathematical representation of a biochemical system does not have to start from scratch. In fact, the history of quantitatively studying chemical and biochemical processes is almost 200 years old. Three major roots of today's understanding of biochemical processes and networks are thermodynamics, kinetics, and stoichiometry. We summarize some key findings of these disciplines, and proceed by identifying approaches that offer a good balance between validity, justifiability, interpretability, and mathematical effectiveness and efficiency.

Thermodynamics

Biological systems are based on physical principles. They must satisfy the laws of physics just like any other entity in the physical world. Among the laws and principles of physics, the results of thermodynamics are of particular interest for chemical and biochemical processes, because they govern the relationships between mass, energy, work, and heat. They describe which processes are possible and which are energetically infeasible. As Callen (1960) put it, "Thermodynamics is the study of the macroscopic consequences of myriads of atomic coordinates, which, by virtue of statistical averaging, do not appear explicitly in a macroscopic description of a system." Many theoretical results of thermodynamics were first observed in experiments addressing questions that related energy to pressure, temperature, and volume in ideal gases. Only later were the results formulated as laws and corollaries. "The basic problem of [classical] thermodynamics is the determination of the equilibrium state that eventually results after the removal of internal constraints in a closed composite system" (Callen 1960, p. 7). A simple example is the observation that coffee, if left to its own devices, after a while assumes room temperature.

A significant finding of thermodynamics is that systems tend toward states of minimal energy. In terms of biological systems, this is quite counterintuitive, because organisms apparently violate these laws all the time. Plants generate high-energy compounds like complex sugars out of lower-energy substrates like CO_2 and water, which contrasts simple thermodynamic arguments that would postulate the opposite, namely the degradation of high-energy compounds into lower-energy compounds. Even though organisms have tasks that require increases in energy, they cannot simply ignore or violate the laws of thermodynamics but instead must find ways to complete their tasks in spite of them.

Many books have been written on thermodynamics, some by celebrated scientists such as Max Planck (1945) and Enrico Fermi (1956). These treatises address the topic from a purely physical viewpoint and often use mathematical concepts of considerable sophistication. A reasonably intuitive introduction to the area from a biological perspective is Jou and Llebot (1990); the discussion below more or less follows this book. Katchalsky and Curran (1967), Westerhoff and van Dam (1987), and Heijnen (2001) also treated the topic in the context of biological processes. Two more general texts are Callen (1960) and Kestin (1966).

The first law of thermodynamics asserts that the change in energy within a closed system, which does not gain or lose matter, is equivalent to the sum of heat and of energy-increasing work that the system receives from the outside. Hermann von Helmholtz (1821–94) formulated this law as the widely acknowledged impossibility of constructing a *perpetuum mobile*, a machine of perpetual motion (of the first kind) that would indefinitely produce more energy than it received (Gerthsen and Kneser 1971).

The second law further limits possible transitions within a system. It asserts that heat does not pass spontaneously from a cold to a hot body, unless other processes are in effect. Such a transition would be possible according to the first law, according to which the overall state of energy remains constant, but is not possible according to the second law. The second law is not only valid for isolated systems but also can be formulated to include more realistic, nonisolated systems. Such systems are closed with respect to matter, but exchange heat and work with the environment. The vast majority of biological systems are nonisolated and, furthermore, open with respect to the flux of matter. Their openness presents a significant challenge for thermodynamic considerations.

One reformulation of the second law of thermodynamics is based on Gibbs' (1839–1903) concept of *free energy*, which is defined as the sum of the internal energy of a state of the system and the product of pressure and volume, from which the product of temperature and entropy is subtracted. The entropy characterizes the current state of the system and, in some sense, is a measure of the disorder or statistical homogeneity of its molecules (see Callen 1960, Appendix B for intuitive explanations of the concept of entropy). Highly structured systems have low entropy, whereas unstructured systems have high entropy. In this terminology, the second law of thermodynamics states that the change in Gibbs free energy must be less than zero for any spontaneous process in a closed system that operates under constant pressure

and constant temperature. Expressed differently, the entropy in such a system cannot decrease and the order cannot increase.

Instead of supposing that pressure and temperature are constant, other variables, such as the internal energy of the system and the volume could be considered constant. Each set of variables that are assumed to be constant yields a different constraint in the other variables, and these constraints are called *thermodynamic potentials*. In closed systems, the different thermodynamic potentials are in a sense equivalent, and each contains all the thermodynamic information about the systems, such as specifications of the equilibrium state to which the system moves, including stability of this state, characteristics of phase changes, and relationships between the various thermodynamic quantities.

If expressing similar phenomena in different ways seems confusing, be consoled by Callen (1960, p. 85), "The peculiar multiplicity of formulation and reformulation of the basic thermodynamic formalism is responsible for the apparent complexity of a subject which in its naked form is quite simple." This simplicity stems from the fact that essentially all thermodynamic constraints are computed as partial derivatives of one and the same function, which describes the energy status of the system.

If the system is open, the thermodynamic potentials are not only functions of temperature, volume pressure, and free energy, but also of the numbers of moles of each chemical species present. The change in energy that accompanies a change in the number of moles is called the *chemical potential*. The chemical potentials of all species (types of molecules) relate to the internal energy of the system in a fashion analogous to temperature. For instance, two connected systems exchange energy until both attain the same temperature, and two connected compartments containing different chemical species move toward molecular homogeneity. In both cases, the systems move toward the state of maximal entropy, and one can show that this state corresponds to one of minimal energy. This has significant implications for the characterization of such phenomena as osmotic pressure, diffusion, freezing point depression, and boiling point elevation. The tendency of systems toward a state of lower energy can be observed macroscopically. As early as 1872, Ludwig Boltzmann (1844–1906) furthermore showed that this tendency could be explained in terms of *microstates*, which correspond to locations and properties of individual molecules. The use of probabilistic arguments for predictions of transitions between macrostates has led to the subfield of statistical thermodynamics.

Alberty (1994) reviewed thermodynamic concepts for systems consisting of chemical and biochemical reactions. The equilibrium in such a system is affected by the ionic milieu and typically changes if the pH is altered. Ion effects on changes in free energy are particularly important for reactions involving nucleic acids, proteins, and other polyelectrolytes. To account for these effects, Alberty (1994, 2000) proposed to include the pH as an additional independent variable in thermodynamic computations, just like temperature, volume, pressure, and chemical potentials, and to redefine fundamental quantities like the Gibbs free energy and entropy correspondingly.

Although classical thermodynamics has been called "one of the outstanding achievements of the scientific mind" (Katchalsky and Curran 1967), it is limited

in scope, because it provides a theory for systems that are either in equilibrium or are undergoing reversible processes. Idealized physical systems may satisfy these requirements, but biological systems rarely do so. Organisms are nonisolated and open, regularly exchanging not only heat, but also matter. Equilibrium states and transitions between them, which constitute the cornerstone of classical thermodynamics, are not as relevant for an organism, because equilibrium would mean death. Instead, biological systems operate at *nonequilibrium stationary states*. These states are characterized by influxes and effluxes of matter and energy that are in balance and keep the concentrations of all chemical species (more or less) constant over time. Such states can only be maintained by an external supply of energy.

Nonequilibrium thermodynamics thus deals with questions of energy that are linked to transport and metabolism in open, *dissipative* systems, which require the influx of energy. These systems usually contain *irreversible* processes, which Callen (1960) defines as requiring an increase in entropy. All systems in the real world are of this type. Dissipative systems must overcome the constraints given by the laws of thermodynamics through the coupling of two or more processes. One process leads to a structure with higher energy and lower entropy, thereby running in the direction opposite to the one predicted by its thermodynamic affinity. The energetic gain in this process, which apparently violates the second law of thermodynamics, is "paid for" by energy released in a concomitant reaction that supplies energy and moves in the direction predicted by its thermodynamic affinity. A typical example is the coupling of phosphorylation with oxidation. Phosphorylation is crucial for the storage of chemical energy; probably the most prominent case is the conversion of ADP into ATP. The increase in energy during this process is coupled with energy transfer from another reaction, such as the oxidation of NADH to NAD^+. In animals, the external energy supply is chemical, whereas plants, of course, may also use sunlight for some of the reactions that lead to higher-energy compounds.

Nonequilibrium thermodynamics allows the estimation of energy requirements in dissipative systems, the extent and stoichiometry of reactions or pathways, and the degree of coupling among them. It also characterizes the efficiency of coupled reactions, which is defined as the ratio of free energy consumed in one direction over the energy liberated in the opposite direction. For example, it permits the estimation of the number of moles of oxygen needed for the phosphorylation of one mole of ADP and the computation of the efficiency of photosynthesis. Deductions from the principles of nonequilibrium thermodynamics have also led to the insight that, in the vicinity of the thermodynamic equilibrium, stationary nonequilibrium states are characterized by minimum entropy production (Prigogine 1947/1955). At such a state, the system loses minimal amounts of free energy and is energetically most economical, which might be a rationale for the uncounted control mechanisms with which organisms tend to preserve this state (Katchalsky and Curran 1967). Overall, nonequilibrium thermodynamics provides a collection of constraints that observed or hypothetical reactions in vitro and in vivo must satisfy. Ricard (1999) provides good explanations of the relationships between nonequilibrium thermodynamics and chemical and biochemical rate equations.

An intrinsic feature of the thermodynamic approach is its strict focus on energy, which almost completely excludes time. Thus, a typical result characterizes the energetic possibility or likelihood of a reaction, but it gives no indication whether this reaction occurs on a time scale of seconds or years. Sometimes, temporal considerations are not necessary, but in other cases timing is crucial. If that is the case, thermodynamics is usually not the optimal approach, and one will instead focus on *kinetic* representations of reactions.

Kinetics

Thermodynamics and kinetics are not entirely unrelated. In fact, kinetics may be considered an "empirically based form of generalized thermodynamics" (Westerhoff and van Dam 1987). Kinetics does not focus on energy levels as much as thermodynamics. Instead, it addresses the *temporal* aspects of a reaction. How fast does the reaction proceed? What is the half-life of a metabolite? What affects the speed of the reaction? Certainly, answers to these questions involve thermodynamics at a deeper level, but kinetic studies minimize aspects of energy and instead center directly on metabolite concentrations and fluxes, as well as their fluctuations over time.

In the overwhelming majority of studies, kinetic analyses ignore spatial features. Instead, it is implicitly assumed that all participants of a reaction are available in a homogeneous mix. It is also typically assumed that the substrate concentration is much higher than the enzyme concentration, so that the availability of enzyme drives the process. Some newer studies have questioned some of these assumptions and propose alternative descriptions. We will discuss a few of them throughout the chapter.

The typical chemical or biochemical rate function relates the temporal change in a chemical compound or metabolite concentration to the concentration itself. In the simplest case of a first-order degradation process that does not involve an enzyme, the rate is directly proportional to the concentration. In straightforward notation, this elemental chemical reaction reads

$$v(X) = -kX. \tag{1.2}$$

The rate constant k is positive by definition, because it represents the turnover per time unit, which cannot be negative. The negative sign indicates that material X is actually lost from the existing pool. The mathematical form of the equation results from considerations of statistical thermodynamics that go back more than a hundred years to Svante Arrhenius (1859–1927). Details can be found in textbooks on thermodynamics and kinetics.

Because the rate $v(X)$ represents the change in concentration over time, we can write it as a derivative with respect to time, namely

$$\frac{dX}{dt} = \dot{X} = -kX. \tag{1.3}$$

In kinetic studies, the differentiation variable is almost always time, t, and it is

becoming customary to denote the derivative with the dot notation, as indicated in Eq. (1.3). This notation is somewhat simpler, but does not explicitly identify time as the independent variable.

It might be illustrative to relate the kinetic equation to concepts of statistical thermodynamics. Following the exposition of Westerhoff and van Dam (1987), we consider a simple irreversible reaction

$$\mathrm{A} \rightarrow \mathrm{B} + \mathrm{C}$$

and denote the number of molecules of substrate A at time t with x. From a mechanistic point of view, it is almost impossible to predict when a given substrate molecule will be converted into molecules of types B and C. However, if there are very many molecules of type A, statistical thermodynamics allows us to consider the conversion of any given molecule as a probabilistic process. Because no mechanism replenishes A, the number x can only decrease over time. Specifically, one can formulate the probability of a drop from x to $x - 1$ during a small time interval Δt as

$$P(x, t \rightarrow x - 1, t + \Delta t) = k \cdot x \cdot \Delta t, \tag{1.4}$$

where k is some constant. If Δt is chosen to be small enough, there will be no drop from x to $x - 2$, $x - 3$, etc. within Δt. If we let $P_x(t)$ denote the probability that there are x substrate molecules at time t, we can formulate the probability for x molecules at time $t + \Delta t$ as

$$P_x(t + \Delta t) = k \cdot (x + 1) \cdot \Delta t \cdot P_{x+1}(t) + (1 - k \cdot x \cdot \Delta t) \cdot P_x(t). \tag{1.5}$$

This so-called *master equation* expresses in the first term on the right-hand side the probability of a transition from $x + 1$ to x molecules and in the second term the probability that the number does not fall further from x to $x - 1$. If Δt is decreased toward zero, Eq. (1.5) becomes a set of differential equations, each of which describes the dynamics of a given number x of molecules in the system. These equations can be solved, and one can compute the expected (average) number $\bar{x}$ of substrate molecules at any given time. The result is

$$\bar{x} = x_0 \cdot \exp(-k \cdot t), \tag{1.6}$$

where x_0 is the initial number of substrate molecules. This equation is exactly the solution to the differential equation (1.3), which constitutes the macroscopic, kinetic description of the reaction. The derivation based on master equations demonstrates that the introduction of stochastic elements compensates for our lack of precise knowledge of the molecular state of the system. The details of this derivation and further extensions are found, for instance, in Westerhoff and van Dam (1987).

As an embellishment to the simple degradation of a substrate A, suppose X_1 is converted into X_2 in an elemental chemical reaction. Because no material is lost in this ideal situation, the production of X_2 equals the degradation of X_1, but with opposite sign, because one increases and the other decreases. Thus, we can write the

process as a small system with two variables:

$$\begin{aligned} \dot{X}_1 &= -kX_1 \\ \dot{X}_2 &= kX_1. \end{aligned} \tag{1.7}$$

(Note that *time* does not count as a variable in these types of discussions.) The solution of the linear differential equation (1.3) is an exponential function of time. Thus, the time dependence of X is nonlinear, but the function describing the process corresponds to a differential equation whose right-hand side is linear.

The linear differential equation (1.3) is not only used for elemental chemical reactions. Of relevance to our discussion are simple reactor and chemostat designs (Bailey and Ollis 1977). It is also often assumed as the default for outflow from well-stirred reactors, for generic decay and growth processes, and for standard compartment models (e.g., Edelstein-Keshet 1988; Jacquez 1996).

If two metabolites are involved in a *bimolecular* reaction, their concentrations enter the right-hand side of the (differential equation) rate law as a product. In the generic case where X_1 and X_2 are substrates of such a reaction that generates product X_3, the increase in the concentration of X_3 is given as

$$\dot{X}_3 = k_3 X_1 X_2. \tag{1.8}$$

It is noteworthy that this type of process leads to a *product* in its substrate concentrations and not a sum, even though one might speak of "adding a second substrate" or formulate the reaction as $X_1 + X_2 \rightarrow X_3$. The reason for the product form of the rate law lies partly in thermodynamics and partly in the fact that the two molecules have to come into physical contact. In a homogeneous mixture, the latter is a matter of probability, which suggests as the simplest model a formulation as product.

In the case of Eq. (1.8), the concentration of X_3 increases, and therefore the right-hand side does not carry a minus sign. Notice that X_3 does not appear on the right-hand side. It does not affect its synthesis and depends entirely on the availability of both X_1 and X_2. For every molecule of X_3 that is produced, one molecule of X_1 and one molecule of X_2 disappear. Therefore, the loss in either one substrate is

$$\dot{X}_1 = \dot{X}_2 = -\dot{X}_3 = -k_3 X_1 X_2. \tag{1.9}$$

One may discuss whether there are reactions that truly involve more than two substrates or whether such reactions are in fact sequences of bimolecular reactions. Regardless, theory suggests that such reactions would again be described with differential equations whose right-hand sides consist of products of substrates. For instance, suppose one molecule of X_1 and two molecules of X_2 would be converted into product X_3. The describing rate equations would be

$$\begin{aligned} \dot{X}_3 &= k_3 X_1 X_2^2, \\ \dot{X}_1 &= -k_3 X_1 X_2^2, \\ \dot{X}_2 &= -2k_3 X_1 X_2^2. \end{aligned} \tag{1.10}$$

The exponent 2 associated with X_2 indicates that two molecules are used. It is consistent with the product of X_1 and X_2 in the bimolecular reaction. One says that the process is of (kinetic) order 1 with respect to X_1 and of (kinetic) order 2 with respect to X_2. Note that the rate constant is doubled in the last equation to indicate that X_2 disappears at twice the rate of X_1 and at twice the rate of the synthesis of X_3.

Westerhoff and van Dam (1987) pointed out that kinetic details of elemental processes could always be incorporated in thermodynamic descriptions. As an example, they considered a simple reaction with one substrate S and one product P. The typical kinetic description of the process is some rate function of the two independent variables S and P. However, one could also express the independent variables as log([S]/[P]) and [S] + [P]. The first of these terms is directly related to the thermodynamic concept of the affinity of the reaction, which demonstrates that concentrations can readily be transformed into thermodynamic forces.

The Michaelis–Menten Rate Law

In the early 1900s, Michaelis and Menten (1913) proposed a reaction scheme for enzyme-catalyzed reactions, based on earlier ideas of Henri (1903). They postulated that a substrate S and its catalyzing enzyme E form an intermediate complex (ES) in a reversible reaction. Once formed, this complex would break apart and either return substrate and enzyme or yield product P while simultaneously releasing the enzyme molecule unchanged. The typical diagram including rate constants for the process is shown in Figure 1.4. In this simple form, synthesis of product from the intermediate complex is assumed to be irreversible; in other words, there is no reaction with rate k_{-2}.

Equipped with rate equations for bimolecular reactions (between substrate and enzyme), the formulation of equations is straightforward. One obtains

$$\dot{S} = -k_2(ES), \tag{1.11a}$$

$$(\dot{E}S) = k_1 S \cdot E - (k_{-1} + k_2) \cdot (ES), \tag{1.11b}$$

$$\dot{P} = k_2(ES). \tag{1.11c}$$

The easiest way to understand the equations is to go backward. The equation for the synthesis of product P is a simple elemental reaction with rate k_2, whose substrate is the intermediate complex (ES). The change in the concentration of the intermediate complex is governed by three processes: the bimolecular reaction involving substrate S and free enzyme E; the reverse reaction with rate k_{-1}, in which the intermediate complex (ES) breaks down into enzyme and substrate; and the forward reaction with rate k_2 that leads to the generation of product. Stoichiometry between substrate and product and the quasi-steady-state assumption discussed next require the change in substrate to be the negative of the synthesis of product.

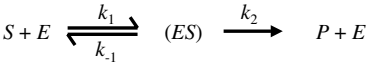

Figure 1.4. Diagram of an enzyme-catalyzed reaction mechanism according to Henri, Michaelis, and Menten.

The system of equations describes the conversion of substrate into product over time. Its main assumption is that the laws of elemental chemical kinetics apply, which require that the reaction proceeds in a homogeneous mixture and that enough substrate and enzyme are available to justify the probabilistic arguments underlying the theory of chemical kinetics. The differential equations can be solved with a numerical integrator, such as the Runge–Kutta or Gear algorithms, which are available in standard software packages for mathematical analysis.

Further assumptions are necessary to obtain the familiar form of the (algebraic) Michaelis–Menten rate law. Like many authors, Schulz (1994, Chapter 1, p. 8) lists the following four

Assumption 1: $E_T = E + (ES)$
Assumption 2: $S_T \gg E_T$
Assumption 3: $\dot{E}_T = (\dot{E}S) = 0$
Assumption 4: $P_0 = 0$

The first assumption is an *enzyme conservation expression* indicating that the total enzyme concentration, E_T, can be divided into free enzyme and enzyme bound in the intermediate complex. The second assumption states that the total substrate concentration S_T is much larger than the total enzyme concentration E_T. The third statement is called the *quasi-steady-state assumption*. Its first part, $\dot{E}_T = 0$, asserts that no enzyme is formed or lost during the process; it is usually accepted as true, at least in vitro. The second part, $(\dot{E}S) = 0$, is more critical. It presumes that the concentration of the intermediate complex is constant. This assumption is clearly violated at the beginning of the experiment. Furthermore, it implies that the reactions forming and destroying the intermediate complex are much faster than the overall conversion of substrate into product. The fourth assumption is made for convenience and could be dropped. It just says that no product is present at the beginning of the experiment.

The algebraic form of the Michaelis–Menten rate law derives from Eqs. (1.11b) and (1.11c), the quasi-steady-state assumption, and the formulation of $\dot{P}$ as the rate v_p. The result is

$$0 = k_1 S \cdot E - (k_{-1} + k_2) \cdot (ES) \tag{1.12a}$$

$$v_p = k_2(ES). \tag{1.12b}$$

Assumption 1 allows us to replace (ES) with the difference between the constant quantity E_T and E. Simple algebra then leads to the so-called Briggs–Haldane (Briggs and Haldane 1925) formulation of the rate as

$$v_p = \frac{k_1 k_2 E_T S}{k_{-1} + k_2 + k_1 S}. \tag{1.13}$$

It is customary to rearrange the parameters and define the *Michaelis constant*

$$K_M = (k_{-1} + k_2)/k_1 \tag{1.14}$$

and the *maximum velocity*

$$V_{\max} = k_2 E_T. \tag{1.15}$$

With these new parameters, the Michaelis–Menten rate law has the familiar form

$$v_p = \frac{V_{\max} S}{K_M + S}. \tag{1.16}$$

This form of the rate law, along with many generalizations, has been immensely successful in the characterization of enzymes and the analysis of simple pathways in vitro.

As modelers, we must ask whether this formulation is optimal. Following the arguments earlier in the chapter, we should judge the rate law against two criteria: validity and mathematical convenience.

Validity

The Michaelis–Menten rate law was conceived almost one hundred years ago. For several decades afterward, it was more or less accepted as probably true, and its applicability and validity were not much questioned. However, beginning in the 1970s, the critical assumptions leading to the rate law were more thoroughly scrutinized. Doubts arose as to whether the mechanisms that seemed to work well in vitro would also be operational in vivo. Specific questions targeted the alleged homogeneity within the cell, the validity of the steady-state assumption, the ample availability of substrate, and the dependence of the rate law on conditions like the pH (Roberts 1977). The culmination of these doubts might have been the title of an article by Hill, Waight, and Bardsley (1977), "Does any enzyme follow the Michaelis–Menten equation?" Detailed discussions of these issues can be found in Savageau (1992a, 1995a,b) and Schulz (1994).

Schulz (1994, p. 22ff) analyzed Assumptions 2 and 3 in some detail. Assumption 2 requires the substrate to be available in such excess that its concentration is more or less constant. In particular, is supposes that the fraction of substrate bound to enzyme is insignificant throughout the assay. Most in vitro systems probably satisfy this condition. However, if the substrate is a large molecule and the reaction occurs in vivo, Assumption 2 may easily be violated. In this case, it seems to be more reasonable to include in the set of assumptions something like a substrate conservation expression, analogous to Assumption 1. Such an expression complicates the mathematical formulation and results in a rate law of the form

$$v_p = \frac{k_2}{2}[(K_m + A_t + E_t) - \sqrt{(K_m + A_t + E_t)^2 - 4E_t A_t}] \tag{1.17}$$

(Goldstein 1944; Cha and Cha 1965; Reiner 1969, pp. 82–90), which is "not very convenient" and "rather unwieldy" (Schulz, 1994, pp. 24–5).

If the substrate concentration is not available in essentially unlimited amounts, the rate law becomes a function of time. Schulz (1994, p. 25) proposed to simplify the differential equation model with a model of product synthesis that has the

form

$$P(t) = \frac{k_1 k_2 E_T S_T}{k_{-1} + k_2 + k_1 A_T} + \frac{k_1 k_2 E_T S_T}{(k_{-1} + k_2 + k_1 S_T)^2}\left(e^{-(k_{-1}+k_2+k_1 S_T)t} - 1\right). \tag{1.18}$$

This model is approximately quadratic in the millisecond domain and becomes essentially linear in the range of seconds and minutes. Even though noticeably more complex than the original rate law, Schulz warns that this formulation cannot be entirely valid either. The product concentration cannot grow indefinitely, as the approximate differential equations and this solution would predict, but should show sigmoidal, saturated dynamics.

Schnell and Mendoza (1997) pursued a different approach. They developed a closed-form solution of the Michaelis–Menten reaction scheme for the entire time course of substrate. Their result is

$$[S'](t) = \omega([S'_0]\exp(-kt + [S'_0]), \tag{1.19}$$

where ω is the *Omega function* (Wright 1959; Corless et al. 1996), which satisfies the transcendental equation $\omega(x)\exp(\omega(x)) = x$, $[S'] = [S]/K_M$ is the "reduced concentration," and $k = V_{\max}/K_M$ is the first-order rate constant. The subscript zero refers to the initial concentration.

Heinrich and Schuster (1996) and Schnell and Maini (2000) provided good reviews of work treating the quasi-steady-state assumption (QSSA). Earlier analyses by Segel (1988) and Segel and Slemrod (1989) resulted in a succinct condition for the validity of the QSSA in vitro:

$$\frac{[E_0]}{K_m + [S_0]} \ll 1. \tag{1.20}$$

In vivo, this condition tends to break down (Sols and Marco 1970), motivating Segel and Slemrod (1989) to propose a *reverse QSSA*, in which the substrate, rather than the enzyme is in a quasi-steady state with respect to the overall reaction.

Schnell and Maini (2000) asked what might happen if S is *not* much higher than E. They started with elemental chemical kinetics and assumed that the reverse QSSA is valid. The solution for the entire time course then is

$$[S](t) = [S_0]\exp(-k_1[E_0]\,t) + \frac{K_S[S_0]}{[E_0]}(\exp(-k_2 t) - \exp(-k_1[E_0]\,t)), \tag{1.21}$$

$$[(ES)](t) = [S_0](\exp(-k_2 t) - \exp(-k_1[E_0]\,t)), \tag{1.22}$$

where $K_S = k_{-1}/k_1$ is the equilibrium dissociation constant of S from the intermediate substrate–enzyme complex. This solution closely approximates numerical solutions of the original differential equation system describing the mechanistic Michaelis–Menten scheme. Schnell and Maini showed that the reverse QSSA is satisfied if K_M and $[S_0]$ are both much smaller than $[E_0]$.

In summary, the algebraic Michaelis–Menten rate law is very useful and valid if the substrate is not limiting and the QSSA is satisfied. If this is not the case, substrate and product concentrations are functions of time, and the dynamic time courses

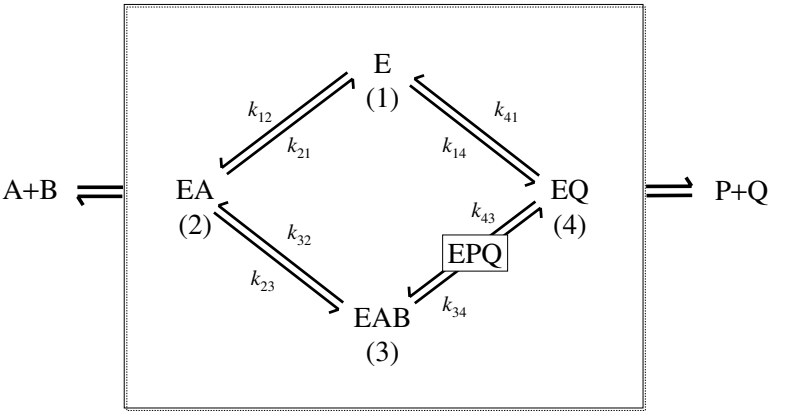

Figure 1.5. Scheme of an ordered bi–bi sequential model of the reaction $A + B \rightleftharpoons P + Q$.

of $P(t)$ and $S(t)$ become complicated. If the reverse QSSA is satisfied, $S(t)$ can be approximated, but the solution is still much more complicated than the original rate law.

Mathematical Convenience

In its original form, the Michaelis–Menten rate law is compact and easy to use, analyze, and interpret. Even some generalizations, for instance accounting for competitive inhibition of the reaction, are easily implemented, and the result retains its simple algebraic character. However, if several substrates or reactions are involved, and if several modulators affect the pathway, the rate law quickly becomes unwieldy.

As an example, consider a reversible *ordered bi–bi sequential reaction* scheme as shown in Figure 1.5. The reaction is called *ordered* because the binding between enzyme and substrates and the dissociation of products from the enzyme occur in a sequential fashion rather than at random. Specifically, the first step binds substrate A and the enzyme, followed by association of the complex EA with substrate B. The three-component complex EAB is converted into EPQ, a process that involves no interaction of the enzyme with a reactant and therefore does not appear in the rate law. Product P must dissociate from the complex before Q. Examples of this mechanism include some pyridine nucleotide dehydrogenases (Schulz 1994, p. 60).

The reaction involves only two substrates and two products, yet the kinetic representation in the tradition of Henri, Michaelis, Menten, Briggs, and Haldane is already quite complicated (Figure 1.6).

If the reaction involves inhibitors and other modulators, the complexity of the rate law becomes overwhelming. Savageau (1976, p. 75) illustrates this with the enzyme glutamine synthetase, which is affected by at least eight reactants and modifiers (Woolfolk and Stadtman 1967). Even under the restrictive assumption that none of the reactants or modifiers enters the rate law with a power higher than one, this rate law would consist of about 500 terms and would take at the order of one hundred million experimental assays to establish. It is hard to imagine that mathematical